



PDF hosted at the Radboud Repository of the Radboud University Nijmegen

This full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/16659>

Please be advised that this information was generated on 2014-11-15 and may be subject to change.

The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water

S.E. Wendelaar Bonga, G. Flik, P.H.M. Balm, and J.C.A. van der Meij

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Nijmegen, The Netherlands

Accepted December 10, 1989

Summary. Branchial chloride cells, which actively take up ions in the gills of freshwater fish, were studied in tilapia (*Oreochromis mossambicus*) exposed to sublethally acidified freshwater. Structural damage of cells, resulting in cell death by necrosis, only occurred transiently, when the reduction of water pH was acute rather than gradual. The most prominent effects of water acidification were the rapid increase in the number of chloride cells and the changes in frequency of the different stages of the chloride cell cycle. In the opercular inner epithelium, a twofold increase in cells occurred 48 h after gradual acidification. Cell density stabilized after 4 weeks at a level 5 times that of control fish. Four transitory stages were distinguished in the chloride cell cycle: accessory or replacement cells, immature, mature, and degenerating (apoptotic) cells. In control fish, mature chloride cells dominated (over 50%) with immature and apoptotic cells totalling about 40%. After 4 weeks in acid water, only 13% of the cells were mature. Immature and apoptotic cells dominated, each representing about 40% of the total number of chloride cells. Mature cells apparently age rapidly under these conditions. Thus, chloride cells turn over quickly in acid water, with a minor increase in ion transport capacity of the gills. This conclusion is supported by the observation that opercular and branchial Na^+/K^+ ATPase activities in treated fish are only 40%–50% higher than in controls.

Key words: Chloride cells – Apoptosis – Gills – Water acidification – *Oreochromis mossambicus* (Teleostei)

This paper deals with maturation and degeneration of chloride cells of fish gills under freshwater conditions. Whereas the ion-extruding activity of the gills of seawater

fish has been elegantly demonstrated to be located in the chloride cells (Foskett and Scheffey 1982), and the importance of these cells is well known, the ion-absorptive function ascribed to these cells in freshwater fish has been shown only indirectly. These cells have been described as rudimentary in freshwater fish (Foskett et al. 1981). However, more recent physiological, biochemical and ultrastructural studies have established a close relationship between freshwater chloride cells and the uptake of monovalent and divalent ions (Payan et al. 1981; Flik et al. 1985a; Mayer-Gostan et al. 1987). Furthermore, these cells show adaptive changes such as cell proliferation when the ion balance is disturbed by reduced water ion levels or by various types of water pollution, including water acidification (McDonald 1983; Leino and McCormick 1984; Laurent et al. 1985; Avella et al. 1987). Few studies describe the structural changes associated with increased activity and turnover of chloride cells in freshwater fish.

Teleost chloride cells are structurally characterized by many small mitochondria and an extensive system of small branching and anastomosing tubules, where ion-transporting enzymes such as Na^+/K^+ -ATPase, Na^+/H^+ -ATPase and transport Ca^{2+} -ATPase are located (Hootman and Philpott 1979; Flik et al. 1985b; Balm et al. 1988); the tubules have a lumen that is continuous with the extracellular space via orifices in the basolateral cell membrane (Sardet et al. 1979; Laurent and Dunel 1980). Vesicles are present between the apical membrane and the tubular system in the apical cytoplasm (Pisam 1981; Laurent 1984). This structure is typical of chloride cells of fish from freshwater and seawater. The presence of accessory cells is considered typical of seawater fish (Dunel-Erb and Laurent 1980; Chrétien and Pisam 1986; Pisam et al. 1988). On the other hand, these cells have been interpreted as immature differentiating chloride cells (Sardet et al. 1979; Hootman and Philpott 1980). In an earlier study on tilapia (Wendelaar Bonga and Van der Meij 1989), we found accessory cells in freshwater fish, albeit in very low numbers. We suggested

Send offprint requests to: Dr. S.E. Wendelaar Bonga, Laboratory of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands

that mature chloride cells differentiate from accessory cells in both freshwater and seawater fish, and that the high frequency of accessory cells in seawater was caused by increased cellular turnover. The higher number of accessory cells was accompanied by a higher incidence of chloride cells that were degenerating by apoptosis. If the structural diversity of the chloride cells is indeed related to the turnover rate of the chloride cells rather than to the ionic composition of the water, the changes typical of fish transferred from freshwater to seawater will also occur when turnover has increased after acidification of freshwater.

We used the African cichlid fish *Oreochromis mossambicus* (tilapia), a hardy euryhaline teleost, that was exposed to sublethal acidification of the water. In a preliminary report, we have shown that this treatment leads to a rapid proliferation of the chloride cells in response to the osmoregulatory stress imposed (Wendelaar Bonga and Dederen 1986).

There have been few studies on the ultrastructure of chloride cells of acid-stressed fish, and these are mainly limited to observations on fish from natural acid water, probably contaminated with aluminum or other toxic cations, or on fish exposed to water with a pH close to the lethal level (Chevalier et al. 1985; Leino et al. 1987). The present observations on tilapia deal with fish exposed to a sublethal pH level that has been shown to represent substantial stress for the fish (reduced growth and reproduction), but that is tolerated for at least 6 months without increase in mortality (Flik et al. 1989).

Materials and methods

Sexually mature male and female tilapia (*Oreochromis mossambicus*; formerly *Tilapia mossambica* and *Sarotherodon mossambicus*) of 15–25 g body weight were obtained from laboratory stock. They were kept in 200 l aquaria containing tap water, at 25°C with a daily 12-h photoperiod. The water was continuously circulated through charcoal filters. The concentrations of the water (in mM) were: Na⁺, 5.0; K⁺, 0.06; Ca²⁺, 0.8; Mg²⁺, 0.2; Cl⁻, 4.2; SO₄²⁻, 0.5; pH, 7.6. Water pH was lowered from pH 7.6 to pH 4.5 by adding dilute H₂SO₄ with a multichannel pump either acutely (in 10 min) or gradually (over 4 h). During acidification, the water was well aerated. During the exposure period, water pH was adjusted with NaOH or H₂SO₄ using Radiometer pH-stat equipment. The ammonia concentration of the water (ammonia secretion increased rapidly during the first few days in acid water) was monitored daily and kept below 0.5 mg/l by replacing some of the water. The concentration of aluminum was determined in a Plasma IL 200 Thermo Electron atomic emission spectrometer, and amounted to 21 µg/l.

Groups of 7 fish were exposed for 48 h or 1, 4 or 6 weeks to pH 4.5, and subsequently anesthetized with methoxyethanol. Blood was collected in heparinized hematocrit tubes from the caudal blood vessels after severing the tail. Plasma osmolarity was determined in a Vogel micro-osmometer. Plasma sodium and calcium were measured by flame photometry (Model IV Auto-analyzer, Technikon). One branchial operculum of each fish was removed and incubated for 1 h in a well-aerated 2 µM solution of 2-(dimethylaminostyryl)-1-ethylpyridiniumiodine (DASPEI), which stains the mitochondria-rich chloride cells (Bereiter-Hahn 1976). After rinsing, the inner opercular epithelium was examined in a Zeiss

fluorescence microscope at a magnification of ×250. Because of the complexity of the gill structure, the number of chloride cells is difficult to estimate. We therefore present the number of chloride cells per surface area of the inner opercular epithelium; this value is considered to reflect total branchial chloride cell numbers (Foskett et al. 1981). Cells were counted in 20 different squares of the opercular epithelium with a total surface area of 5 mm² per fish. The data were statistically analyzed using Student's *t*-test.

Gill filaments were dissected and fixed for electron microscopy as described (Wendelaar Bonga and Van der Meij 1989). Chloride cells were examined in the interlamellar areas of the filamental epithelium. The percentages of accessory, immature, mature, apoptotic and necrotic chloride cells were determined on the basis of the classification of 100 chloride cells per animal, in groups of 6 fish. These percentages were determined for control fish and for fish exposed to acid water for 1 or 4 weeks.

For determination of the Na⁺/K⁺-ATPase activity of opercula and gills, groups of 8 fish (a control group from water of pH 7.6 and a group exposed to pH 4.5 for 4 weeks) of 20 ± 1 g body weight were quickly anesthetized in a TRIS-buffered (pH 7.4) MS-222 solution. The bulbus arteriosus was cannulated and the branchial apparatus was perfused with ice-cold isotonic saline containing heparin (20 U · ml⁻¹) to remove the blood cells from the gills. Additionally, 0.2 mM phenylmethylsulphonyl fluoride, a protease inhibitor, was added to the perfusion fluid to increase enzyme recovery. The epithelium of the gills and the inner epithelium of the opercula was scraped off onto an ice-cold glass plate with a glass microscope slide. The subsequent preparative procedure and the Na⁺/K⁺-ATPase assay were performed as described by Flik et al. (1983).

Results

Freshwater controls

General structure. The gill filament is covered by an epithelium consisting of 2–4 layers of flattened epithelial cells (pavement cells), with occasional mucocyte and many chloride cells. The latter cells are mainly concentrated in the filamental epithelium between the respiratory lamellae. The layers of filamental epithelial cells are separated in many places by lymph spaces that may contain different types of leucocytes.

Chloride cells. Many chloride cells display the structure typical of teleost fish with numerous mitochondria and a well-developed tubular system. The apical membrane

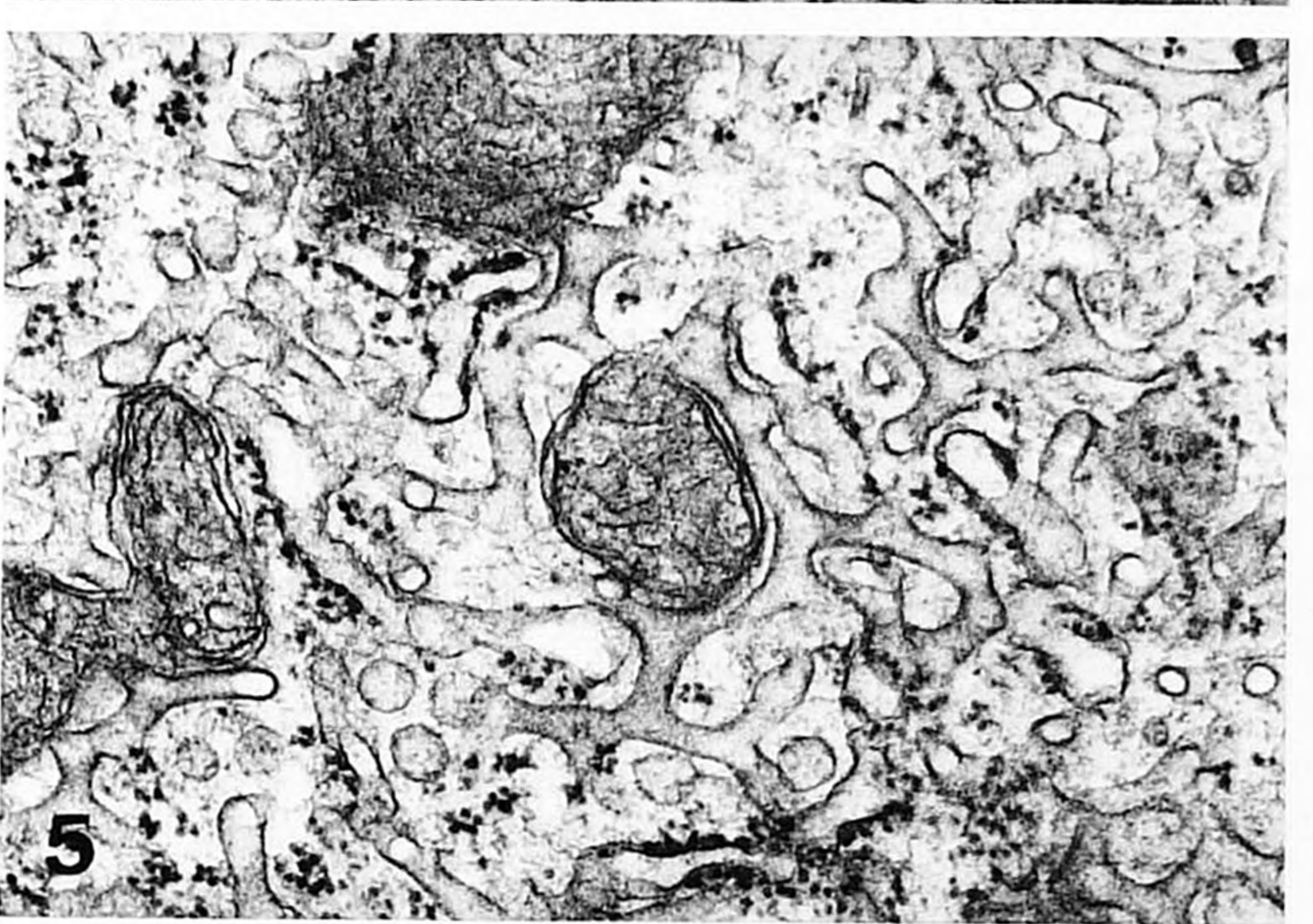
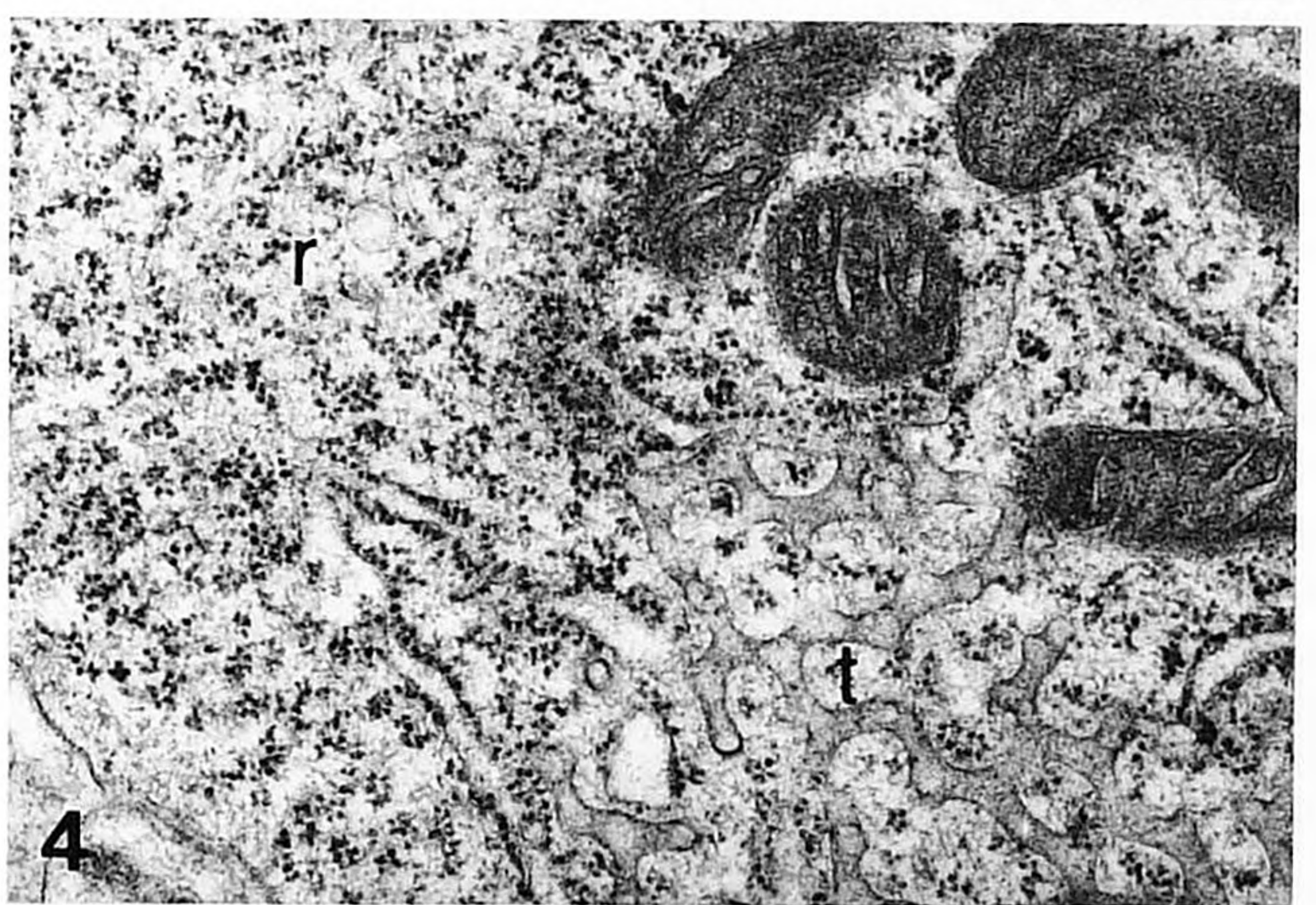
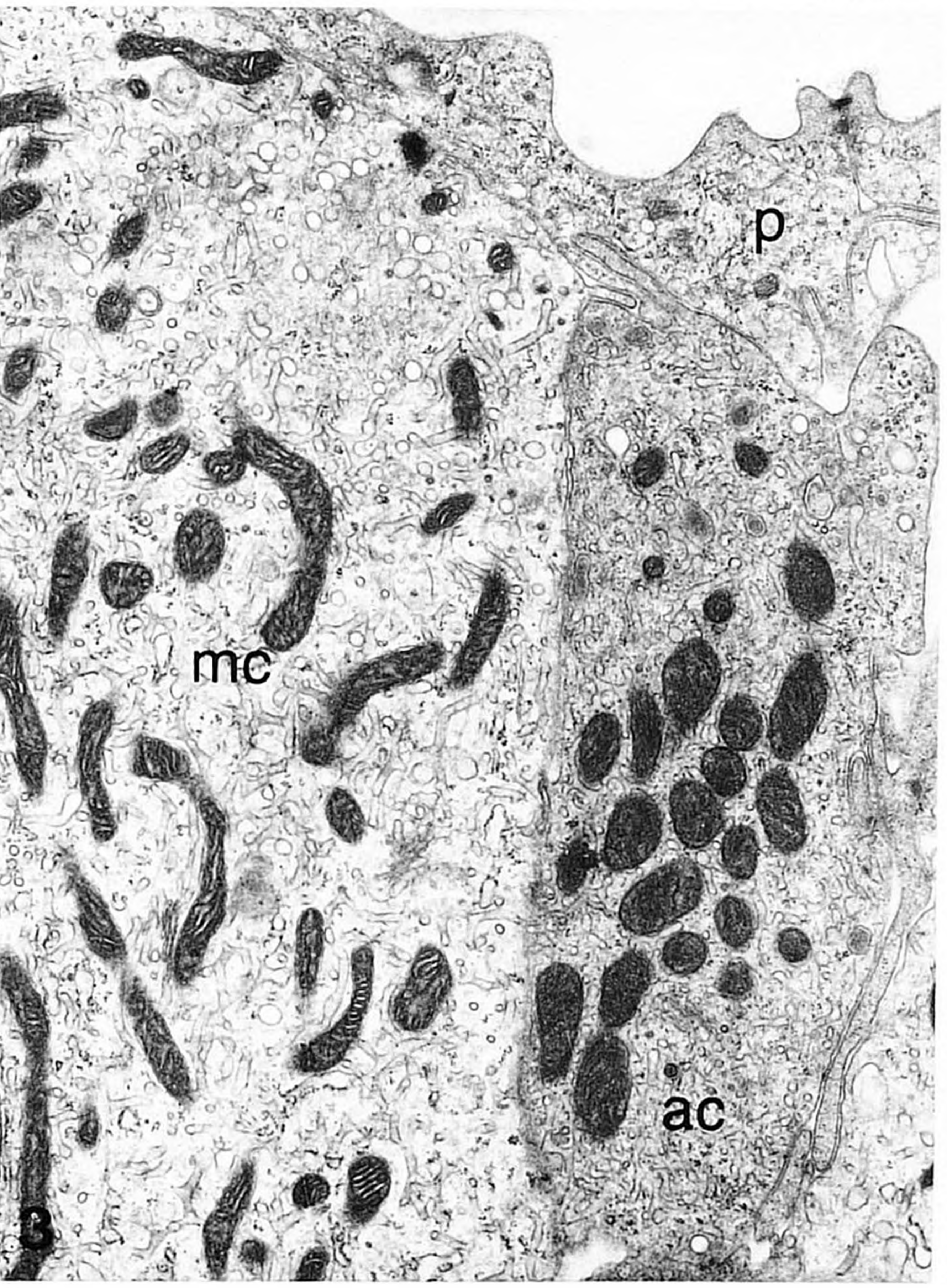
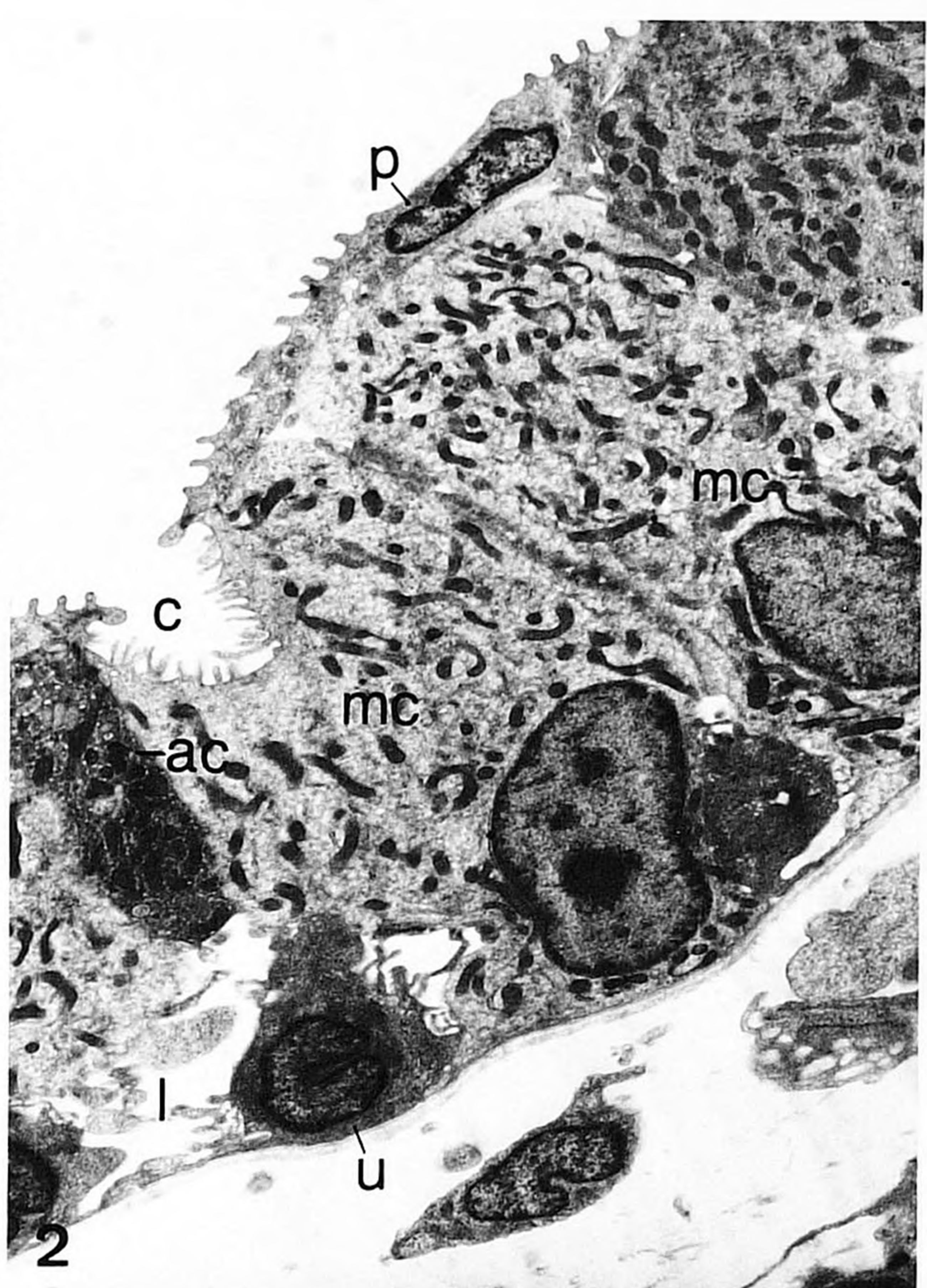
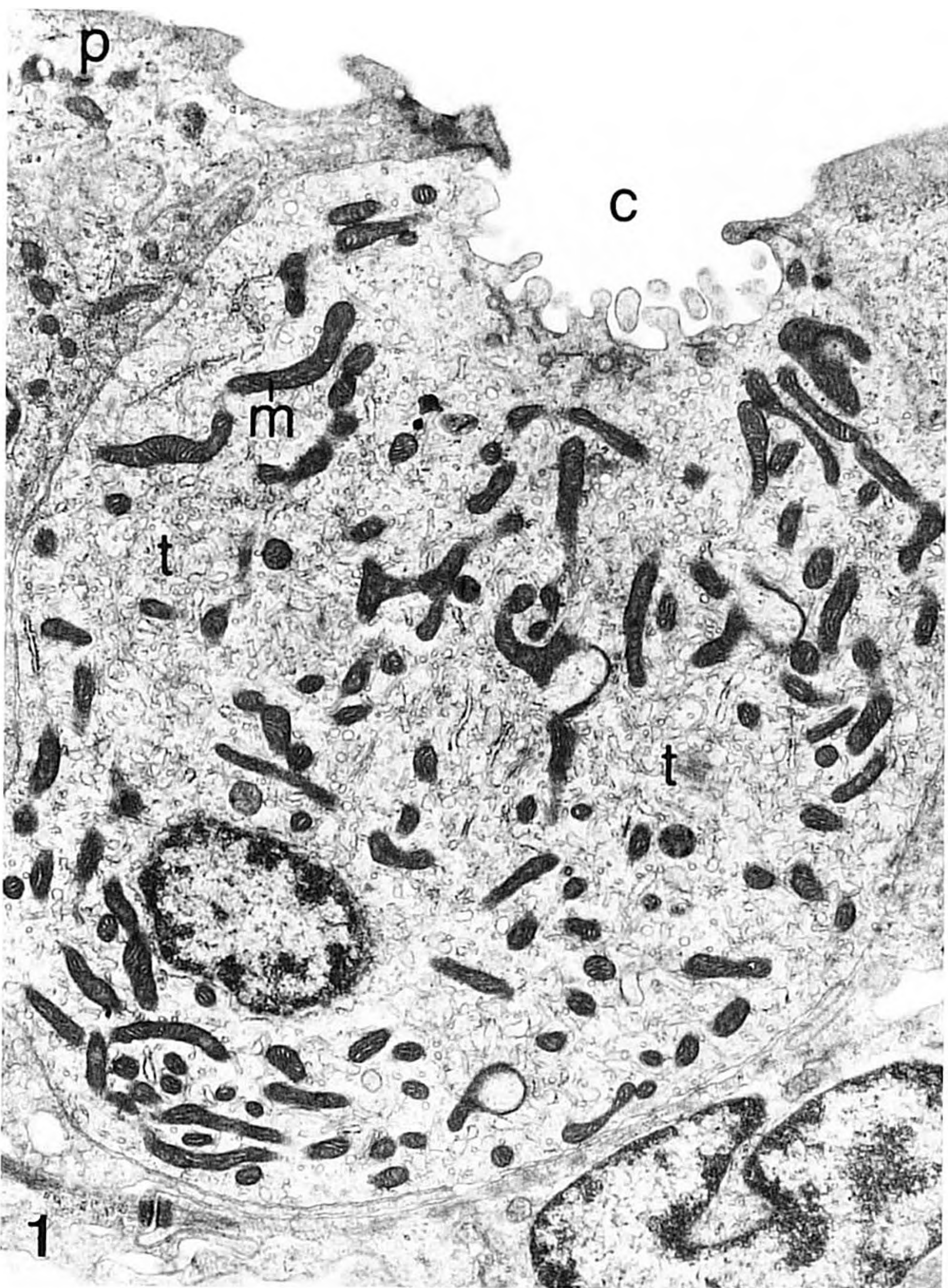
Fig. 1. Filament epithelium, control. Mature chloride cell with tubular system (*t*), mitochondria (*m*), and apical crypt (*c*); *p* pavement cell. × 8500

Fig. 2. Filament epithelium, pH 4.5 (7 days). Mature (*mc*) and apoptotic (*ac*) chloride cells; *c* apical crypt; *l* lymphoid space; *p* pavement cell; *u* undifferentiated cell. × 5500

Fig. 3. Filament epithelium, pH 4.5 (7 days). Mature (*mc*) and accessory (*ac*) chloride cells; *p* pavement cell. × 12500

Fig. 4. Filament epithelium, pH 4.5 (7 days). Part of accessory cell, showing many ribosomes (*r*) and developing tubular system (*t*). × 29000

Fig. 5. Acclimatization to pH 4.5, 7 days. Detail of mature chloride cell with well-developed tubular system and some granular endoplasmic reticulum. × 40000



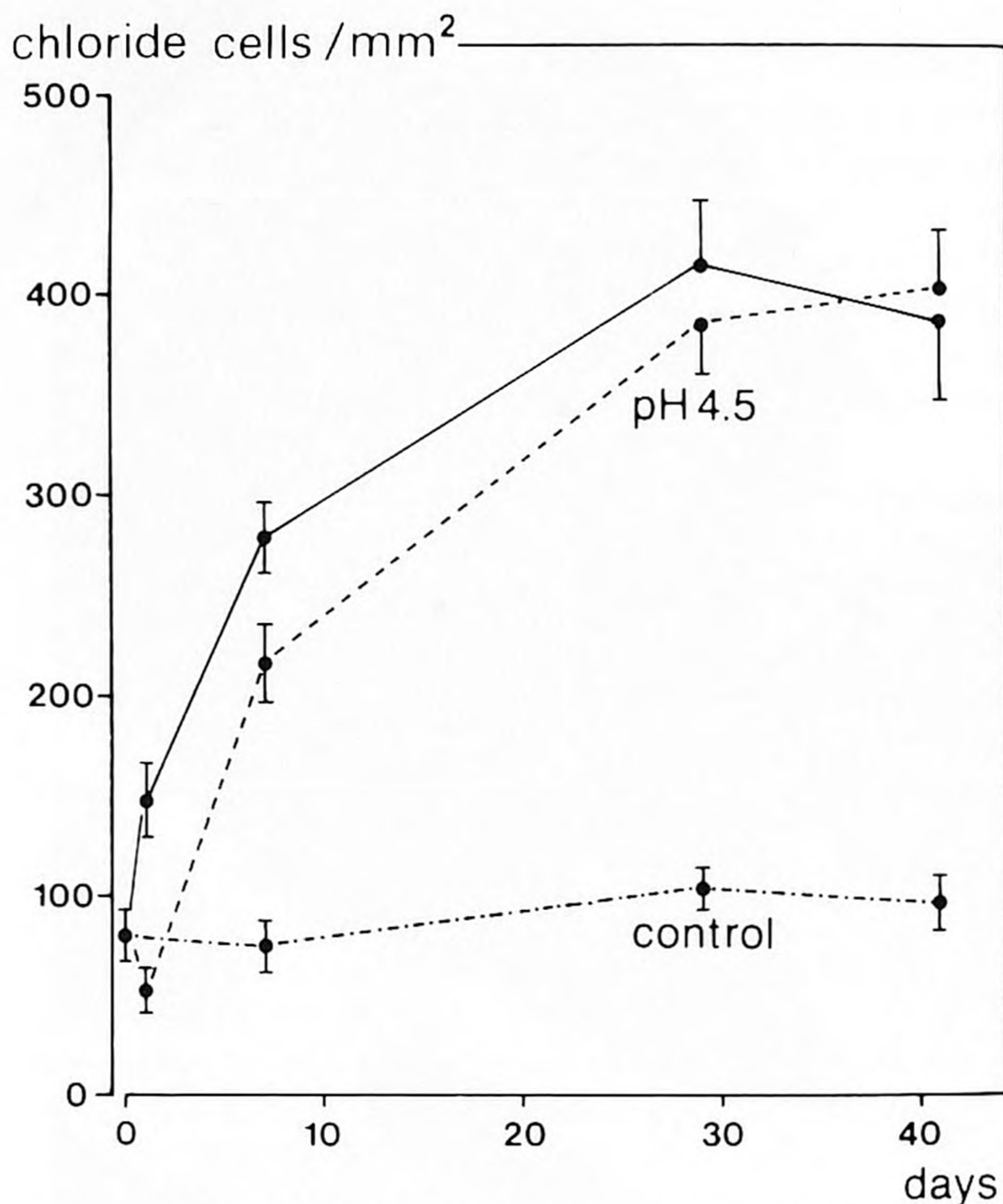


Fig. 6. Density of the chloride cells in the inner opercular epithelium of fish exposed for 2, 7, 28 or 42 days to water of pH 4.5 (*solid line* gradual acidification; *broken line* acute acidification), and of fish from water of pH 7.6 (*control*); means \pm S.D.; $n=7$

of these cells is in contact with the water and may be indented, forming an apical crypt (Figs. 1, 2). This fully differentiated and rounded cell is called a mature chloride cell. It is only one of 4 different stages of the chloride cell cycle, as distinguished in this study. Some of the mature cells are associated with an accessory cell: a slender, often crescent-shaped, small cell, with a less well-developed tubular system and frequently with a denser cytoplasm than the mature chloride cells (Figs. 3, 4). A few chloride cells are structurally intermediate between accessory cells and mature chloride cells. These cells are rounded and often have fully developed apical crypts similar to mature chloride cells, and will be called immature chloride cells. Their granular endoplasmic reticulum is more extensive and the tubular system less developed than in mature cells. Golgi areas, which are scarce in mature and accessory chloride cells, are common; the nuclei are euchromatic with distinct nucleoli. Some chloride cells show signs of degeneration by apoptosis (Wendelaar Bonga and Van der Meij 1989). The cells lose contact with the water and become more dense and rounded. They are finally phagocytosed by macrophages; in these cells, large chloride cell remnants (apoptotic bodies; Wyllie et al. 1981) are found in lysosome-like bodies. In control fish, apoptotic cells are common,

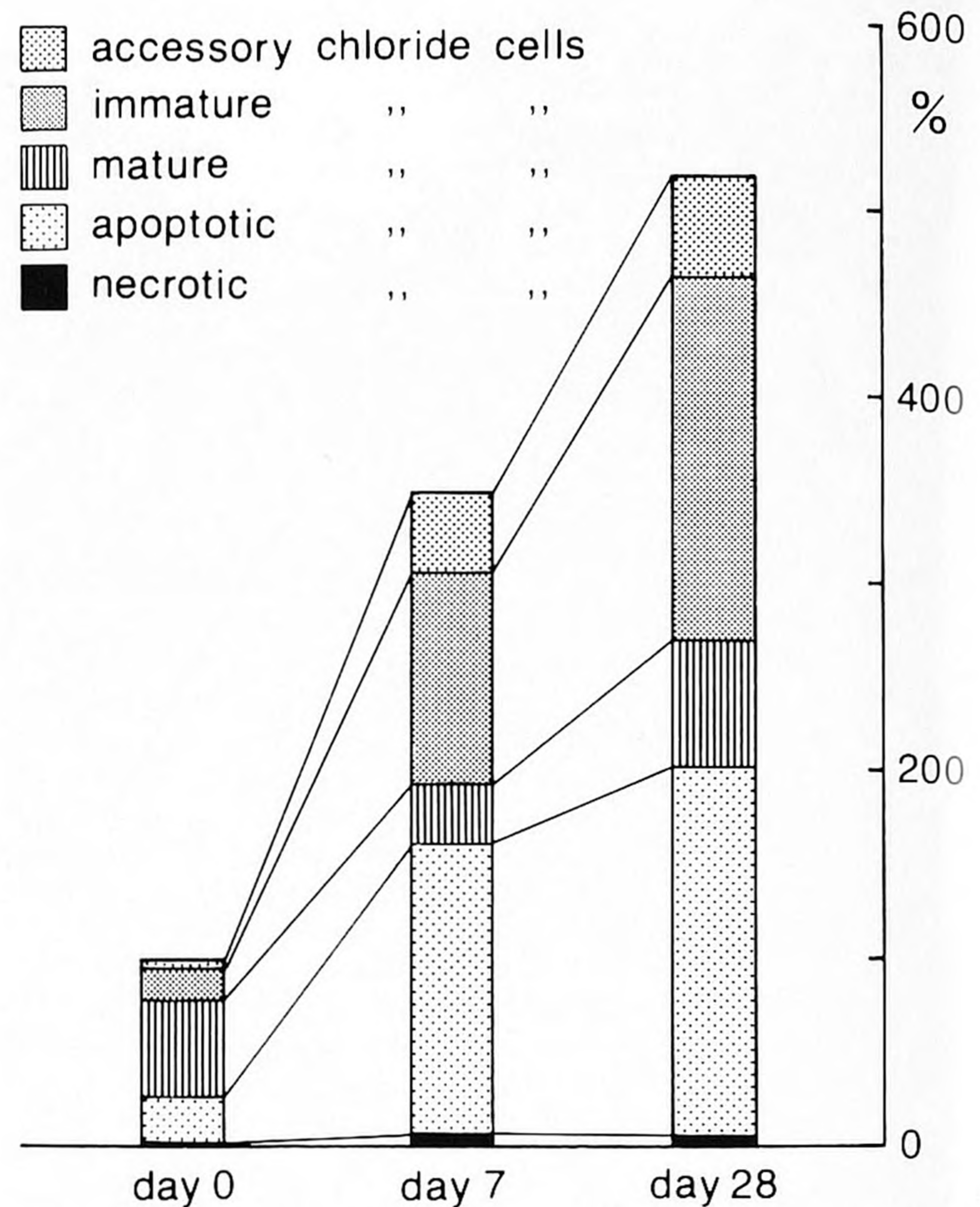


Fig. 7. Relative frequency of the different stages of the chloride cells in control fish (day 0), and fish exposed to water of pH 4.5 for 7 and 28 days, as estimated at the ultrastructural level. The length of the bars reflects the numerical density of the chloride cells as determined in the inner opercular epithelium after DASPEI staining (density of chloride cells in control fish at day 0: 100%)

although the final stages of apoptosis are rarely encountered. This also applies to cell death by necrosis, which is characterized by swelling of the cytoplasm and cytoplasmic organelles and rupture of cellular membranes. The numerical densities of chloride cells determined in the DASPEI-stained inner opercular epithelium of controls and acid-exposed fish are shown in Fig. 6, and mi-

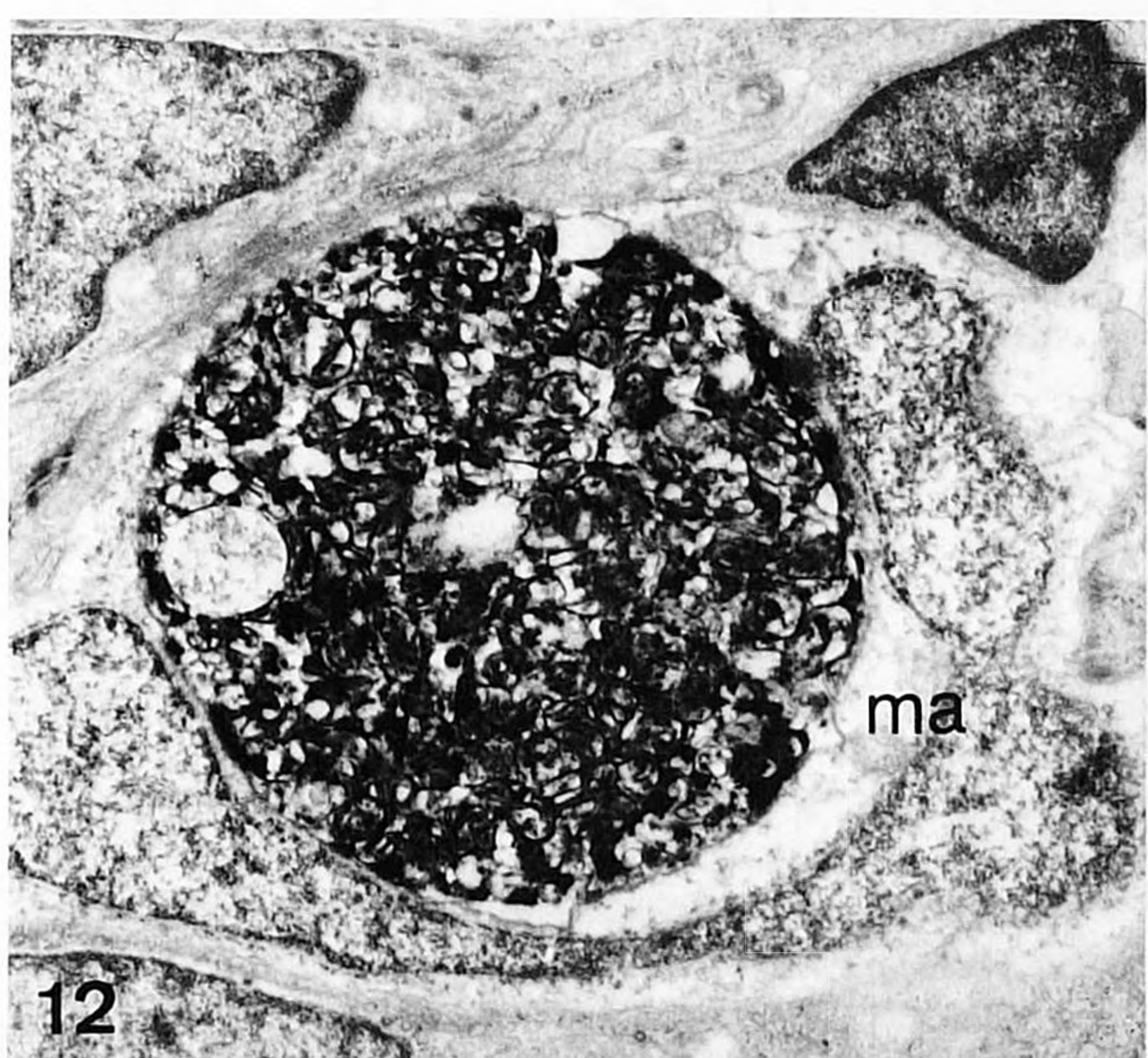
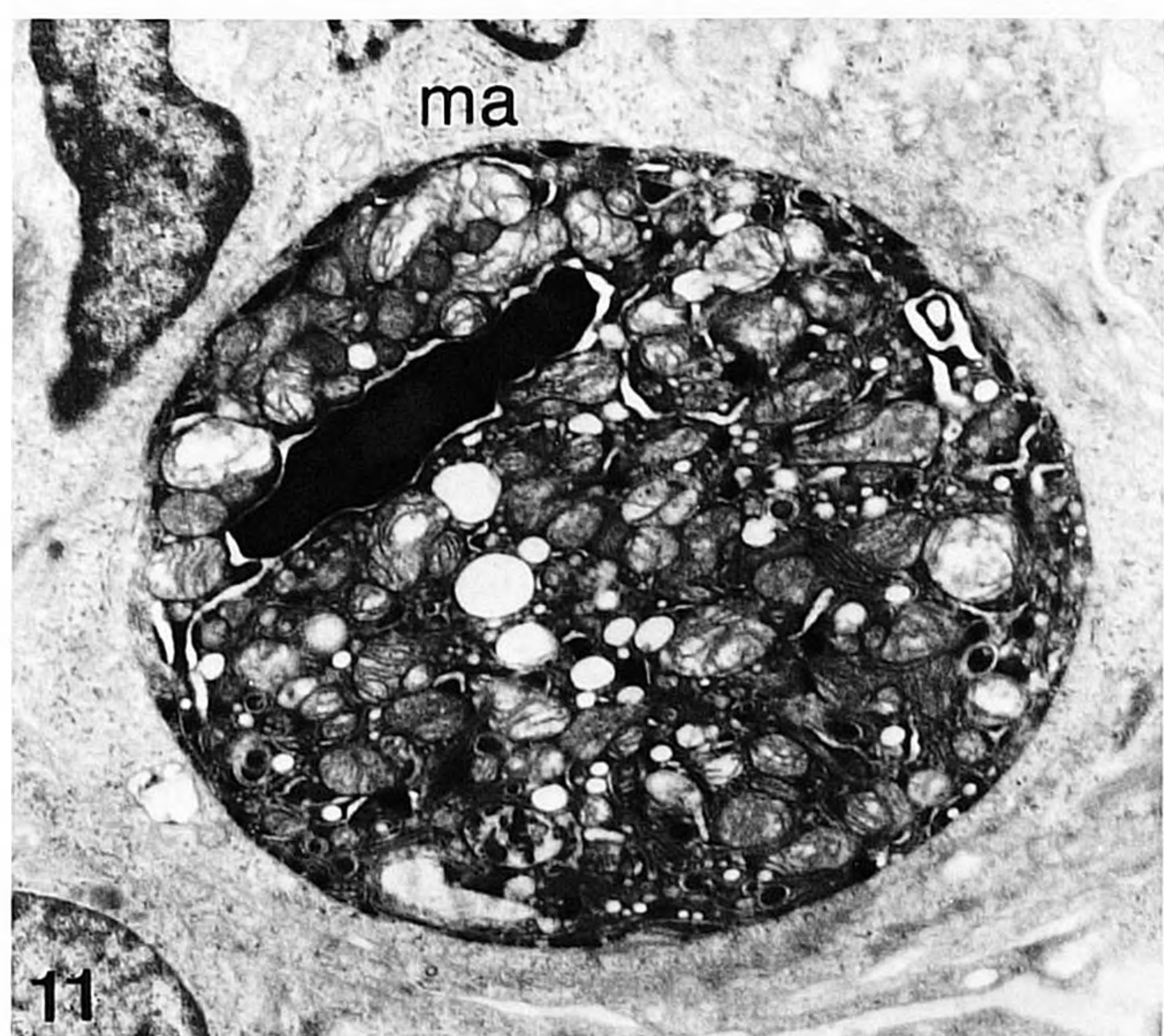
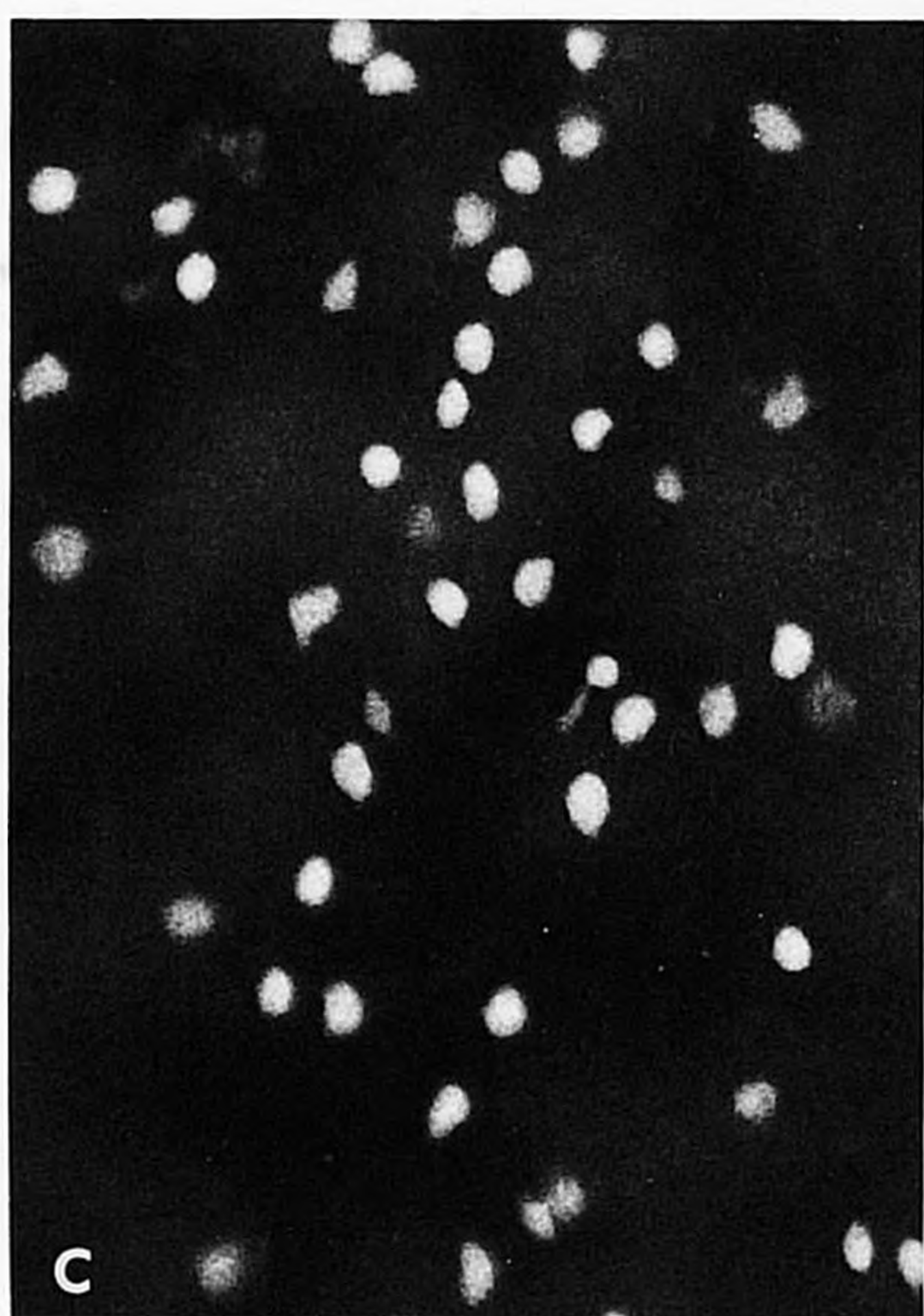
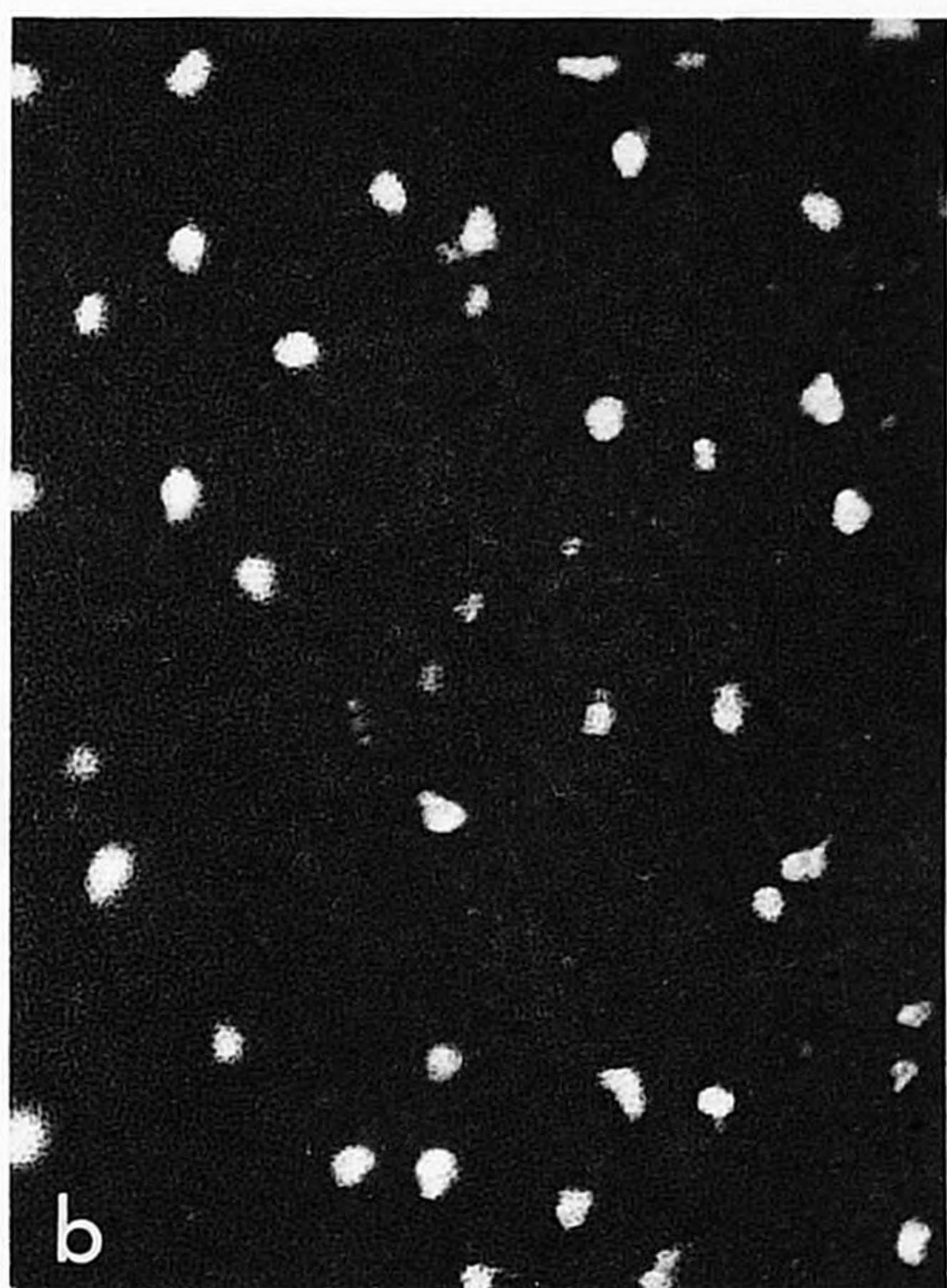
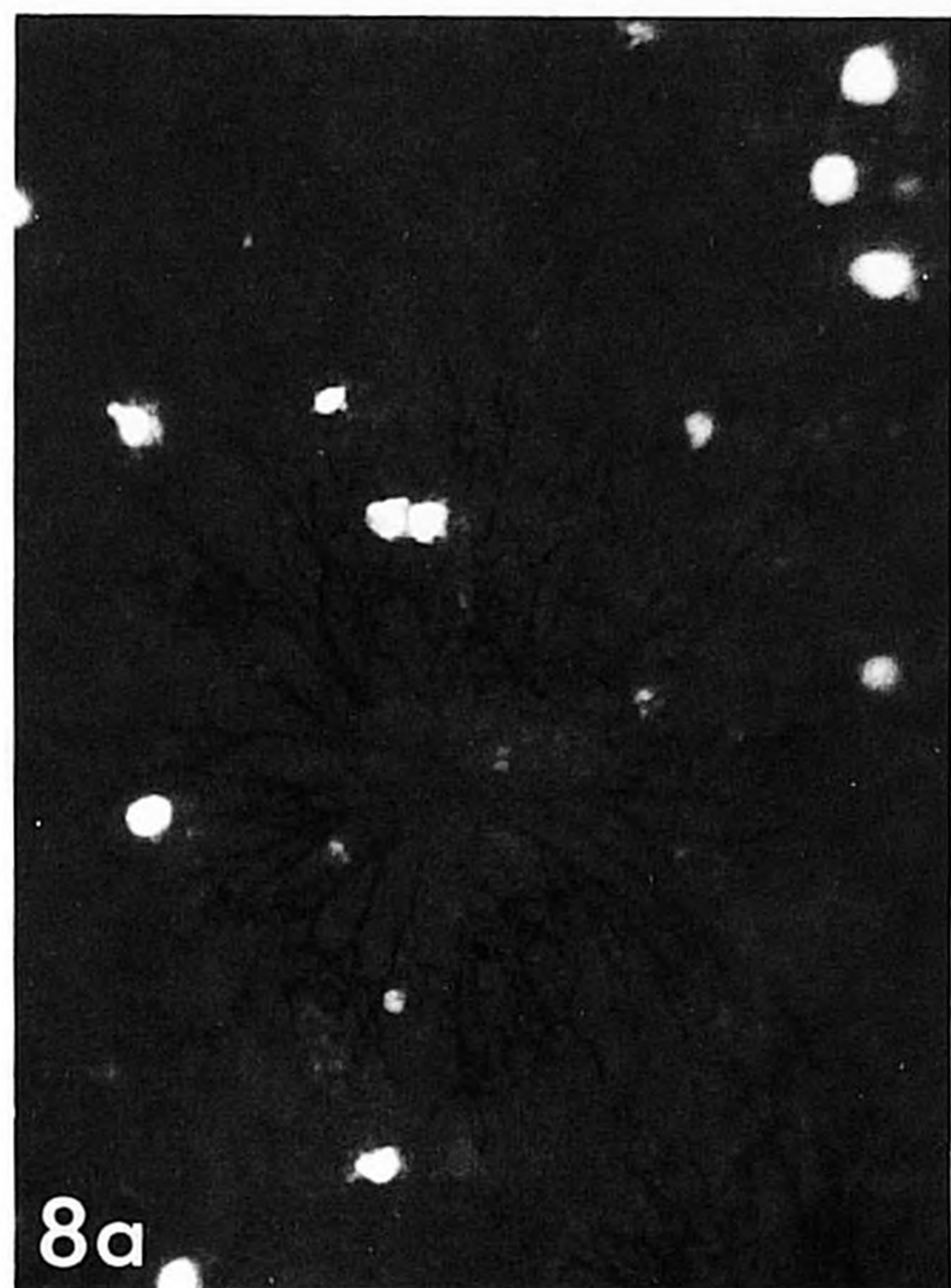
Fig. 8a-c. Micrograph of inner opercular epithelium showing fluorescing chloride cells (DASPEI staining); **a** pH 7.6 (control); **b** pH 4.5, 7 days; **c** pH 4.5, 28 days. $\times 210$

Fig. 9. Acclimatization to pH 4.5, 2 days, acute exposure. Necrotic chloride cell. $\times 9500$

Fig. 10. Acclimatization to pH 4.5, 7 days. Chloride cell showing first signs of apoptosis: loss of apical crypt and cytoplasmic densification. $\times 7200$

Fig. 11. Acclimatization to pH 4.5, 7 days. Apoptotic chloride cell engulfed by macrophage (*ma*); a dense nucleus, dilated elements of the tubular system, and many mitochondria are visible. $\times 12000$

Fig. 12. Acclimatization to pH 4.5, 7 days. Large lysosome-like body, probably representing the apoptotic remnant of a chloride cell, in a macrophage (*ma*). $\times 12000$



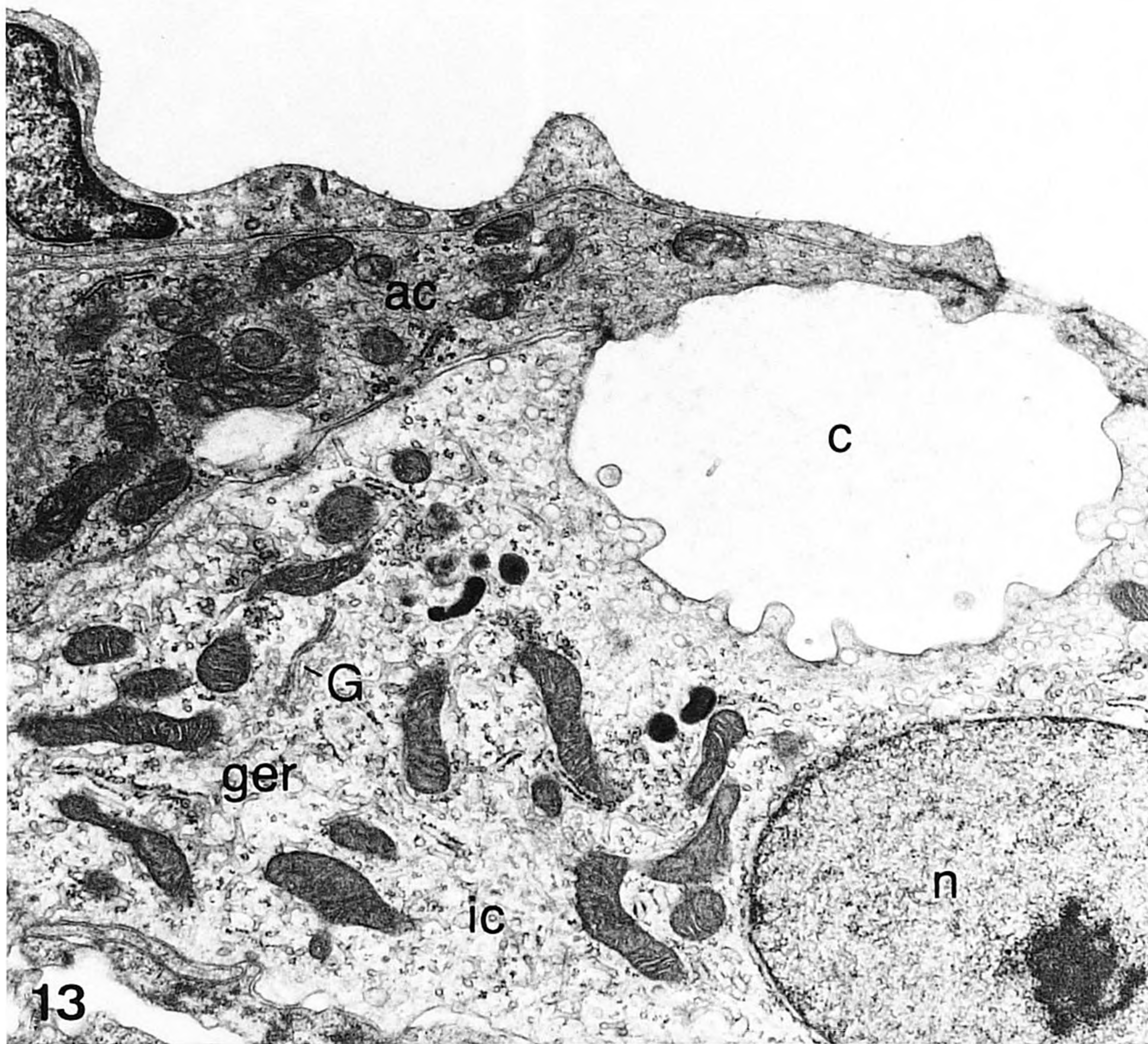


Fig. 13. Filament epithelium, pH 4.5 (7 days). Accessory (*ac*) and immature (*ic*) chloride cells sharing an apical crypt (*c*); the tubular system of both cells is poorly developed; *ger* granular endoplasmic reticulum; *G* Golgi area; *n* euchromatic nucleus with prominent nucleolus. $\times 16000$

crographs of this epithelium in Fig. 8a–c. The relative frequencies of accessory, immature, mature, apoptotic and necrotic cells are shown in Fig. 7.

Acid water

Two days. Exposure to acid water for 2 days induced structural damage in both pavement cells and chloride cells: the incidence of necrosis of these cells, which is uncommon in control fish (less than 1% of the chloride cells) increased (Figs. 7, 9). However, in acid-exposed fish, the frequency of necrosis was strongly related to the initial rate of reduction of water pH. Tissue damage was substantial when the drop in water pH took place in less than 10 min, and comprised 10%–20% of the branchial chloride cells. The percentage of necrotic cells was less than 5% when acidification of the water occurred more gradually (2 h). Under these conditions, the chloride cell density after 2 days was significantly higher ($P < 0.01$) than in the controls and in the fish exposed to acute acidification (Fig. 6). Moreover, the numbers of accessory cells and apoptotic cells notably increased. Mature chloride cells were observed less frequently than in controls (Fig. 7). The lymphoid spaces between the epithelial cells were markedly enlarged when compared to those of the control fish, and contained various types of white blood cells, including macrophages.

As shown in Table 1, plasma osmolarity and sodium

were reduced after 2 days in acutely exposed fish, whereas these parameters were unchanged in the other experimental groups.

One week. The density of opercular chloride cells increased twofold over control values in fish acutely exposed to acidified water. In the fish from gradually acidified water, a threefold increase was found (Figs. 6, 8a, b). Estimates of the number of cells in the interlamellar area indicated a similar increase of chloride cells in the epithelium covering the gill arches (Fig. 2). Ultrastructural examination of the filamental epithelium showed that the percentage of accessory chloride cells had increased to twice that of the controls (about 12%; Figs. 3, 7, 12). The number of immature and apoptotic chloride cells showed a similar increase as the accessory cells, to about 30% and 45%, respectively (Figs. 7, 10–13). However, the number of mature chloride cells amounted to less than 10%, compared with more than 50% in the controls (Fig. 7). In most of the mature chloride cells, the granular endoplasmic reticulum and free ribosomes were more frequent than in the mature cells of control fish (Fig. 5). All phases of apoptosis were commonly found: cells showing the first symptoms of cytoplasmic densification, without apical crypt, but apically in contact with the water (Fig. 10); more condensed cells, with swollen tubular systems that were separated from the water by pavement cells; fully condensed cells, phagocy-

Table 1. Plasma osmolarity, sodium and total calcium levels of control fish and of fish exposed for 2, 7 and 28 days to water of pH 4.5. The water was acidified either acutely or gradually. Means \pm SD of 7 fish per group

	Osmolarity mosmol \cdot l $^{-1}$	Sodium mM	Calcium mM
2 Days			
Controls	317 \pm 8	144 \pm 7	3.06 \pm 0.17
pH 4.5 (acute)	294 \pm 6*	126 \pm 6*	2.94 \pm 0.13
pH 4.5 (gradual)	321 \pm 4	142 \pm 5	3.12 \pm 0.21
7 Days			
Controls	324 \pm 5	146 \pm 4	2.96 \pm 0.16
pH 4.5 (acute)	314 \pm 7	136 \pm 8	3.11 \pm 0.11
pH 4.5 (gradual)	316 \pm 9	142 \pm 5	2.89 \pm 0.18
28 Days			
Controls	314 \pm 7	139 \pm 7	3.12 \pm 0.24
pH 4.5 (acute)	319 \pm 10	145 \pm 11	3.22 \pm 0.18
pH 4.5 (gradual)	316 \pm 11	140 \pm 8	3.02 \pm 0.11

* Significantly different from controls, $P < 0.05$

tosed by macrophages, but still recognizable as chloride cells (Fig. 11); and large electron-dense phagosomes in macrophages, probably representing apoptotic remnants of chloride cells (Fig. 12). Necrosis was rarely observed (Fig. 7).

Plasma osmolarity, sodium and total calcium levels in the acid-exposed fish were not significantly different from the controls.

Four and six weeks. After prolonged exposure to acid water, the chloride cells had further increased in number, and showed a fivefold rise compared with the controls (Figs. 6, 8c). No difference in numerical chloride cell density was noticeable between the fish that were acutely or gradually exposed to acid water. The percentage of accessory chloride cells was also above the control level, although lower than after 1 week in acid water (Fig. 7). The percentage of immature cells had slightly increased and that of the apoptotic cells decreased when compared with values on day 7, but both cell phases dominated (40% each). The percentage of mature cells was similar to that at day 7 and was thus low in comparison with control fish. Necrotic cells were rarely found. No differences were observed between fish exposed for 4 or 6 weeks.

Plasma osmolarity, sodium and total calcium levels of the acid-exposed fish were not significantly different from the control levels (Table 1).

Na^+/K^+ -ATPase activity was determined for the gill arches and for the inner opercular epithelium of fish exposed for 4 weeks to pH 4.5, and of controls (Fig. 14a, b). Specific enzyme activity was not significantly increased, in contrast to total enzyme activity per fish. However, the increase in total enzyme activity was limited to 50% for the gills and to 40% for the opercula. Total enzyme activity of the gills was 5–6 times that of the opercula in both groups.

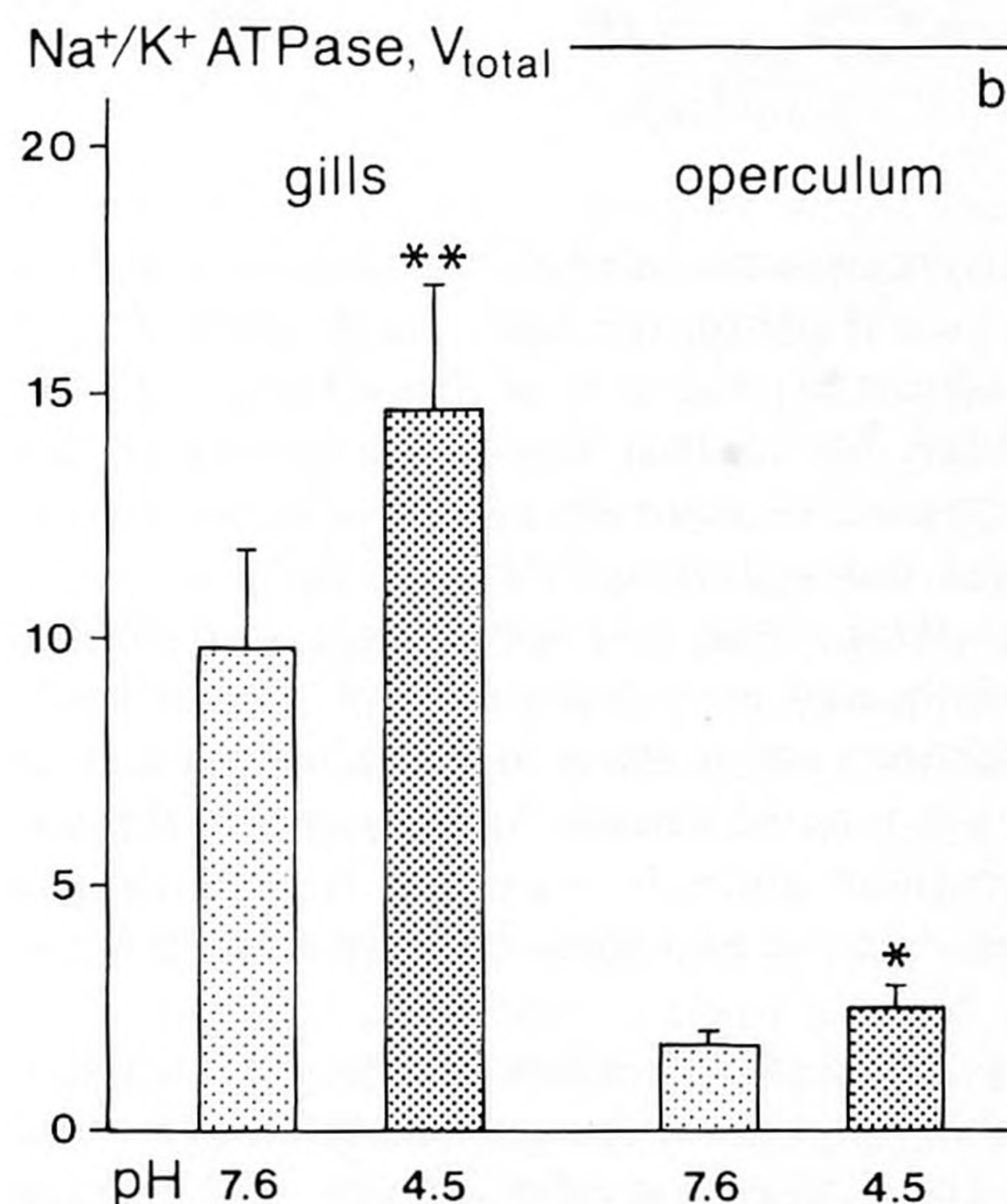
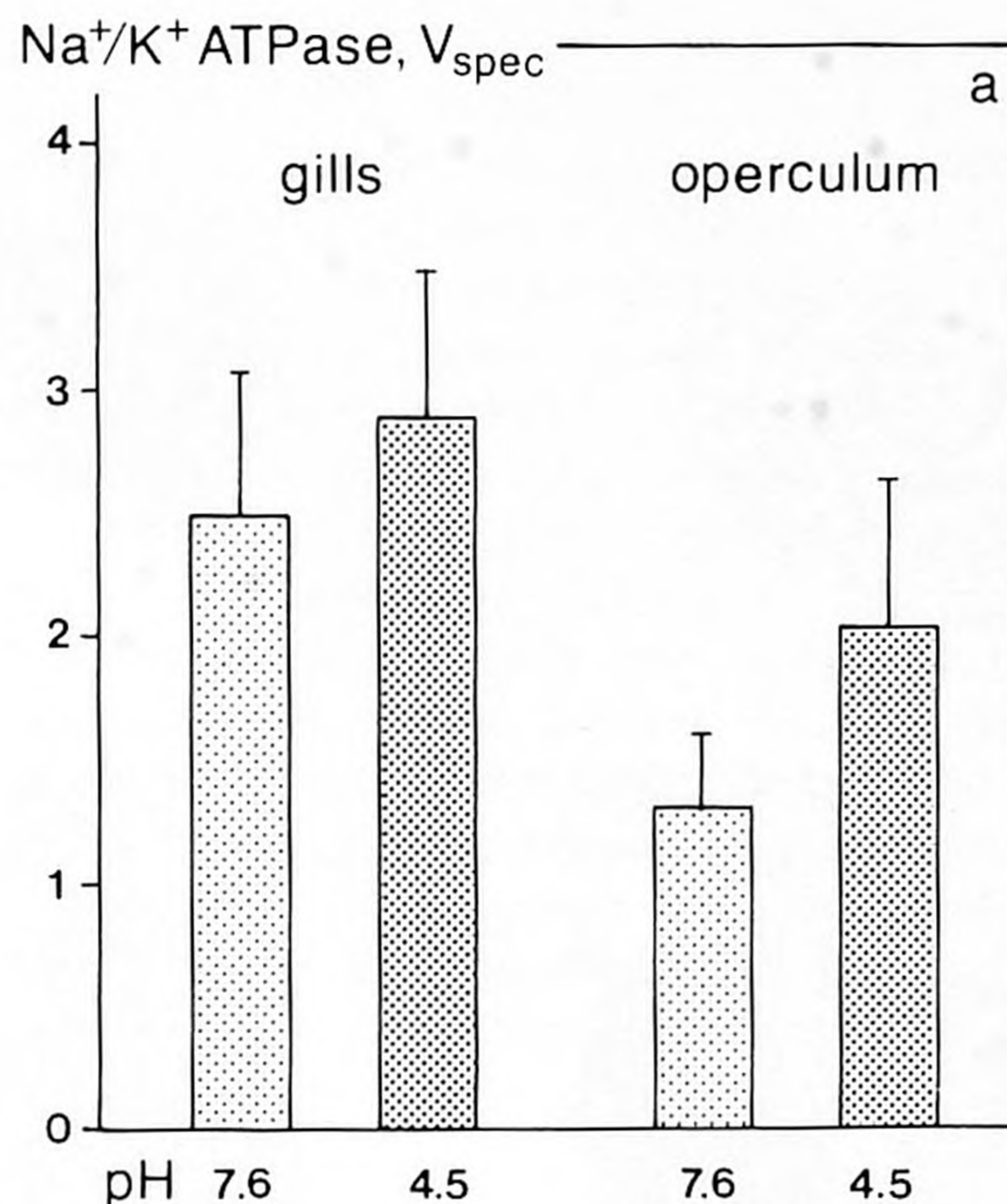


Fig. 14a, b. Specific (a V_{spec} in $\mu\text{mol P}_i \cdot \text{h}^{-1}$ per mg protein) and total (b V_{total}) Na^+/K^+ -ATPase activity of gills and inner opercular epithelia of control fish (pH 7.6) and fish exposed for 4 weeks to acid water (pH 4.5). V_{total} is defined as the product of V_{spec} and total protein per fish (mg) in the initial homogenate of the branchial and opercular epithelia; $n = 8$

Discussion

Water acidity and structural tissue damage

Structural damage of the gills of fish exposed to pH 4.5 is limited. Substantial damage is only found immediately after acute acidification and not after a gradual drop in water pH, and can therefore be ascribed to the method of acidification rather than to low water pH itself. Acute acidification promoted necrotic degeneration of chloride cells, and this may explain the transient reduction in the number of opercular chloride cells observed after 48 h in fish acutely exposed to low pH. After gradual

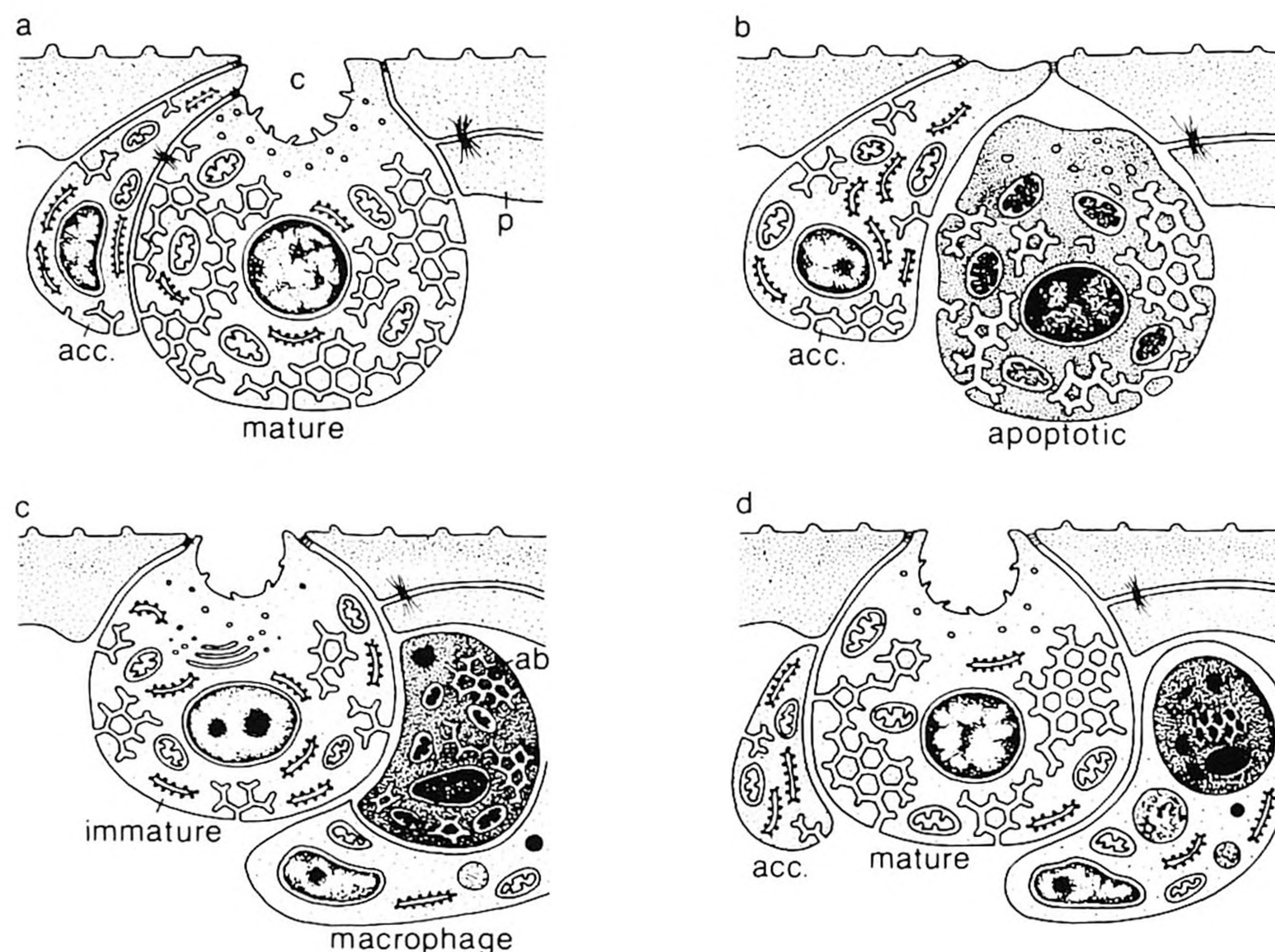


Fig. 15a-d. Diagrammatic representation of the chloride cell cycle; **a** mature chloride cell, associated with an accessory cell (*acc.*); *c* apical crypt; *p* pavement cell; **b** mature chloride cell degenerates apoptotically and loses contact with the water; the accessory cell (*acc.*) develops; **c** immature chloride cell, developed from accessory cell; the apoptotic body (*ab*) of the replaced chloride cell becomes engulfed by a macrophage; **d** new mature chloride cell has developed and is associated with a new accessory cell (*acc.*); the apoptotic chloride cell remnant is digested in the macrophage

acidification, necrosis was infrequent and, in the first few days after acidification, no decrease in chloride cell numbers was observed. After 4 or 6 weeks at pH 4.5, necrosis was back to control levels. Thus, except for the transient increase in necrosis after acute pH reduction, no apparent tissue damage was noticed.

Exposure to pH 4.5 only has minor effects on plasma osmolarity, sodium and total calcium levels. Statistically significant reductions are limited to the acutely exposed fish, and therefore may be caused by the structural damage observed in these animals. Apparently, the fish are able to maintain osmotic and ionic balance after gradual pH reductions.

Few reports on the effects of low pH on gill morphology are available. In line with our results, Jagoe and Haines (1983) and Leino and McCormick (1984) have found that fish, exposed to acidified water above the pH level that causes acute mortality, show little alteration in gill morphology. When the fish are exposed to a pH level that leads to severe osmoregulatory disturbances and mortality, however, chloride cells are damaged and reduced in number (Leino et al. 1987). The severe gill injury reported earlier by Daye and Garside (1976) and Jagoe and Haines (1983) for some salmonid species also concerned fish exposed to lethally acidified water.

The conclusion from the above data, that chronic exposure to sublethal pH levels does not lead to substantial structural damage of the gill epithelium, is seemingly in contrast with the results of Chevalier et al. (1985). Their light- and electron-microscopic observations of gills of brook trout from acidified (pH 5.5) and non-acidified lakes show extensive separation of the epithelial layer from underlying tissues, deformation of lamellae and degeneration of chloride cells. However, as already suggested by the authors, these effects may be attributed

to the high aluminum concentration (more than 10 times that of the water used in our experiments) and the presence of toxic heavy metals in the lake water. Aluminum and heavy metals are known to inflict structural damage to the gills (Mallatt 1985).

Chloride cell density

A prominent effect of acid-water exposure on tilapia is the dramatic increase in numerical density of chloride cells in both the gills and the inner opercular epithelium. This contrasts with some other studies, in which no effect or even a reduction of total chloride cell number have been reported (Chevalier et al. 1985; Leino et al. 1987). However, as mentioned above, these studies involved water acidified to a level that was almost or fully lethal for the species involved, or natural water containing substantial amounts of aluminum and, possibly, other toxic metals. Cell proliferation may have been balanced by cell death under these conditions. In agreement with this suggestion, Leino and McCormick (1984) have reported chloride cell proliferation for fathead minnows in water at sublethal pH and at low concentrations of toxic metals. In the same species, no increase or even a decrease in cell numbers has been noticed at lower pH and in the presence of toxic metals (Leino et al. 1987).

Ultrastructure of the chloride cells

We have identified accessory, immature, mature and apoptotic chloride cells; we consider these to be successive stages of the chloride cell cycle (Fig. 15). During exposure to acid water, the frequency of the different stages changes remarkably.

Accessory cells. Accessory cells have been considered either as developing stages of chloride cells (Hootman and Philpott 1980; Pisam 1981) or as a specific cell-type typical for seawater fish (Sardet et al. 1979; Laurent and Dunel 1980; Chrétien and Pisam 1986). Lacy (1983), Leino and McCormick (1984), Chrétien and Pisam (1986) and Pisam et al. (1989) have reported the presence of an occasional accessory cell in freshwater fish. We have also found accessory cells in freshwater tilapia, and observed that they become more numerous after transfer of the fish from freshwater to seawater (Wendelaar Bonga and Van der Meij 1989). The present data show that accessory cells also become more numerous in tilapia exposed to acidified freshwater. This has previously been reported by Leino and McCormick (1984) in their study on fathead minnows. In tilapia, the percentage of accessory cells is correlated with the rate of increase of the total number of chloride cells: a peak is found 2 and 7 days after the start of the experiment, when total chloride cell density increases rapidly. This relationship supports the interpretation of these cells as young stages rather than as a specific type of chloride cell. It is possible that there are different types of accessory cells, since several authors have reported that, in seawater fish or euryhaline seawater-adapted fish, apical cytoplasmic processes of accessory cells interdigitate with the apical cytoplasm of neighboring mature chloride cells (Sardet et al. 1979; Dunel and Laurent 1980; Hwang and Hirano 1985; Chrétien and Pisam 1986; Pisam et al. 1988; Hwang 1988). Neither Leino and McCormick (1984), nor our group have observed this type of interdigitation in fathead minnows or tilapia, respectively. The name "replacement cells" may be more appropriate than accessory cells for these species.

Immature chloride cells. Especially at days 7 and 28, a high percentage of cells show structural characteristics intermediate between those of accessory chloride cells and mature chloride cells. Although the tubular system is still not fully developed in these cells, the apical differentiation suggests that they are functional, although probably at a low level.

Mature chloride cells. Whereas mature chloride cells are the most common cell stage in control fish, they become scarce in acidified water. The percentage reduction of this cell stage indicates that they age more rapidly in acid water than in water of neutral pH. Nevertheless, the fourfold reduction of the percentage of these cells is compensated for by the fivefold increase in total chloride cell numbers; this results in a larger total number of mature cells in fish from acid water than in control fish.

Apoptotic cells. Apoptosis, the most common type of physiologically controlled cell degeneration and death, has been described for many cell types in the higher vertebrates (Wyllie 1981; Bursch et al. 1985). In fish, it has been reported in cells producing hatching enzyme in pike embryos (Schoots et al. 1983), and in endocrine

cells of the Stannius bodies in tilapia (Wendelaar Bonga and Pang 1986). In an earlier study, we interpreted the progressive densification of the cells, resulting in the formation of large globular structures, as signs of apoptotic degeneration (Wendelaar Bonga and Van der Meij 1989). Such dense apoptotic bodies have also been reported by Daoust et al. (1984) in chloride cells of rainbow trout exposed to mercury and copper. In general, apoptotic bodies of epithelial cells are phagocytosed and destroyed either by neighboring epithelial cells or macrophages (Wyllie 1981). We have only found the involvement of macrophages in this process, both in the present and an earlier study (Wendelaar Bonga and Van der Meij 1989), although in rainbow trout the pavement cells may occasionally phagocytose apoptotic bodies of chloride cells (unpublished observations). The high incidence of apoptotic chloride cells in fish from acid water is further evidence that mature cells age rapidly when exposed to acid water.

Branchial ion transporting activity

Although the chloride cells of acid-exposed fish show a fivefold increase in number, analysis of the ultrastructure of these cells suggests that the increase in ion-transporting activity of the gills is limited: most of the cells are immature or degenerating. The numerical density of mature chloride cells in the inner opercular epithelium is only slightly higher than in the controls. Since in immature cells the tubular system is poorly developed, and since most accessory and degenerating cells are no longer in contact with the ambient water, the ion-transporting capacity of these cells is considered to be low or absent, respectively.

This inference is supported by our observation that, after 4 weeks of acclimatization to acid water, there is only a 40%–50% increase in Na^+/K^+ -ATPase activity per fish, substantially less than might have been expected on the basis of the increase in cell number. Because this increase is mainly accounted for by accessory, immature, and apoptotic cells, we conclude that the Na^+/K^+ -ATPase activity of these cells is low or absent. Hootman and Philpott (1980), using a cytochemical technique, have not been able to detect Na^+/K^+ -ATPase activity in accessory cells. Thus, the total ion transporting capacity of the gill area has not increased to the extent that might be inferred from the fivefold increase in numerical chloride cell density. This agrees with our recent observation that sodium uptake rates (and also diffusional sodium losses) of tilapia exposed for 6 months to water of pH 4.5 are lower than in controls (Flik et al. 1989). In these fish, the chloride cell density of the inner opercular epithelium is four times that of the controls (unpublished observations).

The above data indicate that the ion-transporting capacity of the gills is related to the total number of mature chloride cells rather than to the total number of chloride cells. This limits the value of data obtained with DASPEI staining or with other fluorescent dyes

used for chloride cell quantification that demonstrate mitochondria-rich cells. A high density of mitochondria is also found in accessory and immature cells still lacking a well-developed tubular system. The total number of fluorescing cells is therefore not directly related to the ion-transporting capacity of the gills.

The large number of immature and degenerating chloride cells suggests a dramatic increase in cellular turnover. The functional lifetime of a mature chloride cell is probably more limited in acid water. This interpretation is supported by our earlier observations on chloride cells of tilapia during and after transfer of the fish from freshwater to seawater (Wendelaar and Van der Meij 1989). In general, the changes observed are qualitatively similar to those reported in this study: more accessory cells and immature cells, and a higher incidence of cellular degeneration by apoptosis. Tondeur and Sargent (1979) have shown, by tritiated thymidine labeling, that the turnover of chloride cells of eels in seawater is twice that in freshwater. The structural diversity of the chloride cells seems to be primarily determined by the rate of cellular turnover.

References

- Avella M, Masoni A, Bornancin M, Mayer-Gostan N (1987) Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. *J Exp Zool* 241:159–169
- Balm P, Goossen N, De Rijke S, Wendelaar Bonga S (1988) Characterization of Na^+ -ATPases in gills of freshwater tilapia. Evidence for branchial Na^+/H^+ (NH_4^+)-ATPase activity in fish gills. *Fish Physiol Biochem* 5:31–38
- Bereiter-Hahn J (1976) Dimethylaminostyrylmethylpyridiniumiodine as a fluorescent probe for mitochondria in situ. *Biochem Biophys Acta* 423:1–14
- Bursch W, Taper HS, Lauer B, Schulte-Hermann R (1985) Quantitative histological and histochemical studies on the occurrence and stages of controlled cell death (apoptosis) during regression of rat liver hyperplasia. *Virchows Arch [B]* 50:153–166
- Chevalier G, Gauthier L, Moreau G (1985) Histopathological and electron microscopic studies of gills of brook trout, *Salvelinus fontinalis*, from acidified lakes. *Can J Zool* 63:2062–2070
- Chrétien M, Pisam M (1986) Cell renewal and differentiation in the gill epithelium of fresh- or salt-water-adapted euryhaline fish as revealed by [^3H]-thymidine autoradiography. *Biol Cell* 56:137–150
- Daoust PY, Wobeser G, Newstead JD (1984) Acute pathological effects of inorganic mercury and copper in gills of rainbow trout. *Vet Pathol* 21:93–101
- Daye P, Garside E (1976) Histopathologic changes in surficial tissues of brook trout, *Salvelinus fontinalis* (Mitchill), exposed to acute and chronic levels of pH. *Can J Zool* 54:2140–2155
- Dunel-Erb S, Laurent P (1980) Ultrastructure of marine teleost gill epithelia: SEM and TEM study of the chloride cell apical membrane. *J Morphol* 165:175–186
- Flik G, Wendelaar Bonga SE, Fenwick JC (1983) Ca^{2+} -dependent phosphatase and ATPase activities in eel gill plasma membranes I. Identification of Ca^{2+} -activated ATPase activities with non-specific phosphatase activities. *Comp Biochem Physiol* 76B:745–754
- Flik G, Fenwick JC, Kolar Z, Mayer-Gostan N, Wendelaar Bonga SE (1985a) Whole body calcium flux rates in cichlid teleost fish *Oreochromis mossambicus* adapted to freshwater. *Am J Physiol* 249:R432–R437
- Flik G, Van Rijs JH, Wendelaar Bonga SE (1985b) Evidence for high-affinity Ca^{2+} -ATPase activity and ATP-driven Ca^{2+} transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus*. *J Exp Biol* 119:335–347
- Flik G, Van der Velden JA, Seegers HCM, Kolar Z, Wendelaar Bonga SE (1989) Prolactin cell activity and sodium fluxes in tilapia (*Oreochromis mossambicus*) after long-term acclimation to acid water. *Gen Comp Endocrinol* 75:39–45
- Foskett JK, Scheffey C (1982) The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* 215:164–166
- Foskett JK, Logsdon CD, Turner T, Machen TE, Bern HA (1981) Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. *J Exp Biol* 93:209–224
- Hootman SR, Philpott CW (1979) Ultracytochemical localization of Na^+ , K^+ -activated ATPase in chloride cells from the gills of a euryhaline teleost. *Anat Rec* 193:99–130
- Hootman SR, Philpott CW (1980) Accessory cells in teleost branchial epithelium. *Am J Physiol* 238:R199–R206
- Hwang PP (1988) Multicellular complex of chloride cells in teleost branchial epithelium. *Am J Physiol* 238:R199–R206
- Hwang PP, Hirano H (1985) Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. *J Exp Zool* 236:115–126
- Jagoe JH, Haines TA (1983) Alterations in gill epithelial morphology of yearling sunapee trout exposed to acute acid stress. *Trans Am Fish Soc* 112:689–695
- Lacy ER (1983) Histochemical and biochemical studies of carbonic anhydrase activity in the opercular epithelium of the euryhaline teleost, *Fundulus heteroclitus*. *Am J Anat* 166:19–39
- Laurent P (1984) Gill internal morphology. In: Hoar WS, Randall DJ (eds) *Fish physiology*, vol 10a. Academic Press, New York, pp 73–183
- Laurent P, Dunel S (1980) Morphology of gill epithelia in fish. *Am J Physiol* 238:R147–R159
- Laurent P, Höbe H, Dunel-Erb S (1985) The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. *Cell Tissue Res* 240:675–692
- Leino RL, McCormick JH (1984) Morphological and morphometrical changes in chloride cells of the gills of *Pimephelas promelas* after chronic exposure to acid water. *Cell Tissue Res* 236:121–128
- Leino RL, McCormick JH, Jensen KM (1987) Changes in gill histology of fathead minnows and yellow perch transferred to soft water or acidified soft water with particular reference to chloride cells. *Cell Tissue Res* 250:389–399
- Mallatt J (1985) Fish gill structural changes induced by toxicants and other irritants: a statistical review. *Can J Fish Aquat Sci* 42:630–648
- McDonald DG (1983) The effects of H^+ upon the gills of freshwater fish. *Can J Zool* 61:691–703
- Mayer-Gostan N, Wendelaar Bonga SE, Balm PHM (1987) Mechanisms of hormone actions on gill transport. In: Pang PKT, Schreibman MP (eds) *Vertebrate endocrinology: fundamentals and biomedical implications*, vol. 2. Academic Press, Orlando, pp 211–238
- Payan P, Mayer-Gostan N, Pang PKT (1981) Site of calcium uptake in the freshwater trout gill. *J Exp Zool* 216:345–347
- Pisam M (1981) Membranous systems in the “chloride cells” of teleostan fish gill; their modifications in response to the salinity of the environment. *Anat Rec* 200:401–414
- Pisam M, Prunet P, Boeuf G, Rambourg A (1988) Ultrastructural features of chloride cells in the gill epithelium of the Atlantic salmon, *Salmo salar*, and their modifications during smoltification. *Am J Anat* 183:235–244
- Pisam M, Prunet P, Rambourg A (1989) Accessory cells in the gill epithelium of the freshwater rainbow trout *Salmo gairdneri*. *Am J Anat* 184:311–320

- Sardet C, Pisam M, Maetz J (1979) The surface epithelium of teleostean fish gills. Cellular and junctional adaptations of the chloride cell in relation to salt adaptation. *J Cell Biol* 80:96–117
- Schoots AFM, Evertse PACM, Denucé JM (1983) Ultrastructural changes in hatching-gland cells of pike embryos (*Esox lucius* L.) and evidence for their degeneration by apoptosis. *Cell Tissue Res* 229:573–589
- Tondeur F, Sargent JR (1979) Biosynthesis of macromolecules in chloride cells in the gills of common eel, *Anguilla anguilla*, adapting to seawater. *Comp Biochem Physiol* 62B:13–16
- Wendelaar Bonga SE, Dederen LHT (1986) Effects of acidified water on fish. *Endeavour* 10:198–202
- Wendelaar Bonga SE, Pang PKT (1986) Stannius corpuscles. In: Pang PKT, Schreibman MP (eds) *Vertebrate endocrinology: fundamentals and biomedical implications*, vol. 1. Academic Press, Orlando, pp 439–464
- Wendelaar Bonga SE, Van der Meij JCA (1989) Degeneration and death, by apoptosis and necrosis, of the pavement and chloride cells in the gills of the teleost *Oreochromis mossambicus*. *Cell Tissue Res* 255:235–243
- Wyllie AH (1981) Cell death: a new classification separating apoptosis from necrosis. In: Bowen ID, Lockshin RA (eds) *Cell death in biology and pathology*. Chapman and Hall, London, pp 9–34
- Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251–306